



Immunostimulatory RNA Blocks Suppression by Regulatory T Cells

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Abstract: The role of immune suppression by regulatory T (Treg) cells in the maintenance of immune homeostasis is well established. However, little is known about how Treg cell function is inhibited on viral infection to allow the development of a protective immune response. As viral RNA is a crucial mediator for activation of antiviral immunity, we examined the effects of immunostimulatory RNA and infection with RNA viruses on Treg cell function. We show that synthetic RNA oligonucleotides potently inhibit Treg cell-induced suppression in a sequence-dependent manner. This effect is entirely dependent on TLR7 activation of APCs and subsequent IL-6 production. In addition, stimulation with the RNA viruses encephalomyocarditis virus and Sendai virus that specifically activate the RNA-sensing helicases melanoma differentiation-associated gene 5 (MDA-5) and retinoic acid-inducible gene I (RIG-I) also blocks Treg cell function. Interestingly, this effect is seen even in the absence of APCs. Consistent with this, both Treg and T effector cells express RIG-I and MDA-5. Using MDA-5-deficient mice, we demonstrate that the loss of Treg cell function on infection with encephalomyocarditis virus is strictly dependent on MDA-5 expression by Treg cells. Thus, we show in this study for the first time that activation of a RIG-I-like helicase on Treg cells blocks their suppressive function. Regulatory T (Treg) cells play a central role in the suppression of immune reactions and in the prevention of autoimmune responses harmful to the host (1, 2). In particular, Treg cells suppress the activation of naive T effector (Teff) cells by inhibiting TCR-triggered proliferation and differentiation of these cells (3). In the early phase of microbial infection, the suppressive effect of Treg cells must, however, be overcome to enable the generation of an efficient immune response against invading pathogens (4–6). In recent years, it has been shown that several bacterial components can block the suppressive function of Treg cells. Two distinct mechanisms have so far been described: first, bacterial molecules, such as LPS or CpG DNA, can activate APCs to produce inflammatory cytokines that render Teff cells resistant to suppression by Treg cells (7). Alternatively, Treg cells themselves can be directly stimulated: targeting human Treg cells with CpG DNA or with the bacterial lipoprotein Pam3Cys-SK4 abrogates their suppressive function even in the absence of APCs (8–10). The direct action of bacterial components on Treg cells can, however, also have the opposite effect: activation of Treg cells by flagellin enhances their suppressive function (11). Whereas the effect of bacterial ligands on Treg cell suppression has been described, little is known about the regulation of Treg cell function upon viral infection. Although Treg cells are capable of suppressing antiviral immunity (12), most viral infections can be cleared through the induction of an efficient host immune response, demonstrating that Treg cell suppression can be overcome. Viruses are recognized by the innate immune system through host pattern-recognition receptors (13, 14), and several receptors recognizing molecular patterns present within viral RNA have recently been described. Single-stranded RNA from influenza virus initiates the secretion of type I IFN through activation of TLR7 within the endosome (15). In our laboratory, we found that synthetic short RNA oligonucleotides potently activate TLR7 in a sequence-dependent manner (16). Furthermore, we showed that modification of the RNA by phosphorothioate (PTO) linkage of the backbone enhances TLR7 stimulation (17). Viral RNA can also be recognized by cytosolic RNA sensors, the retinoic acid-inducible gene I (RIG-I)-like helicases (18). This family of structurally related proteins includes RIG-I, melanoma differentiation-associated gene 5 (MDA-5) and LGP2. Many RNA viruses, such as the measles virus, the rabies virus, the vesicular stomatitis virus, the Sendai virus, or

the encephalomyocarditis virus (EMCV) induce type I IFN through these receptors (19–22). We have recently identified virally encoded 5'-triphosphate RNA as the natural ligand for the RIG-I receptor (23), whereas MDA-5 recognizes the synthetic long double-stranded RNA polyinosinic-polycytidylic acid [poly (I:C)] on cytosolic delivery (22). Although the detection of viral RNA by pattern recognition receptors leads to strong immune activation, little is known about the effect of RNA ligands on Treg cell-induced immune suppression. We show in this study that RNA oligonucleotides block suppression by Treg cells in a sequence-specific and TLR7-dependent manner through inflammatory cytokines produced by APC. Infection with viruses known to stimulate the cytosolic RNA-sensing receptors RIG-I and MDA-5 blocked Treg cell function in a direct, APC-independent manner. Indeed, we show that Treg cells express RIG-I-like helicases, and activation of the MDA-5 receptor leads to an arrest of their suppressive function. Thus, suppression can be overcome both indirectly by RNA oligonucleotides activating TLR7 on APC and through direct activation of RIG-I-like helicases expressed by Treg cells.

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Immunostimulatory RNA Blocks Suppression by Regulatory T Cells

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The role of immune suppression by regulatory T (Treg) cells in the maintenance of immune homeostasis is well established. However, little is known about how Treg cell function is inhibited on viral infection to allow the development of a protective immune response. As viral RNA is a crucial mediator for activation of antiviral immunity, we examined the effects of immunostimulatory RNA and infection with RNA viruses on Treg cell function. We show that synthetic RNA oligonucleotides potently inhibit Treg cell-induced suppression in a sequence-dependent manner. This effect is entirely dependent on TLR7 activation of APCs and subsequent IL-6 production. In addition, stimulation with the RNA viruses encephalomyocarditis virus and Sendai virus that specifically activate the RNA-sensing helicases melanoma differentiation-associated gene 5 (MDA-5) and retinoic acid-inducible gene I (RIG-I) also blocks Treg cell function. Interestingly, this effect is seen even in the absence of APCs. Consistent with this, both Treg and T effector cells express RIG-I and MDA-5. Using MDA-5-deficient mice, we demonstrate that the loss of Treg cell function on infection with encephalomyocarditis virus is strictly dependent on MDA-5 expression by Treg cells. Thus, we show in this study for the first time that activation of a RIG-I-like helicase on Treg cells blocks their suppressive function. *The Journal of Immunology*, 2010, 184: 939–946.

Regulatory T (Treg) cells play a central role in the suppression of immune reactions and in the prevention of autoimmune responses harmful to the host (1, 2). In particular, Treg cells suppress the activation of naive T effector (Teff) cells by inhibiting TCR-triggered proliferation and differentiation of these cells (3). In the early phase of microbial infection, the suppressive effect of Treg cells must, however, be overcome to enable the generation of an efficient immune response against invading pathogens (4–6). In recent years, it has been shown that several bacterial components can block the suppressive function of

Treg cells. Two distinct mechanisms have so far been described: first, bacterial molecules, such as LPS or CpG DNA, can activate APCs to produce inflammatory cytokines that render Teff cells resistant to suppression by Treg cells (7). Alternatively, Treg cells themselves can be directly stimulated: targeting human Treg cells with CpG DNA or with the bacterial lipoprotein Pam3Cys-SK4 abrogates their suppressive function even in the absence of APCs (8–10). The direct action of bacterial components on Treg cells can, however, also have the opposite effect: activation of Treg cells by flagellin enhances their suppressive function (11).

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The sequence presented in this article has been submitted to the GEO database at the National Center for Biotechnology Information under accession number GSE2389.

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Abbreviations used in this paper: CM, conditioned medium; DC, dendritic cell; DOTAP, N-[1-(2,3-dioleoyloxy) propyl]-N,N,N-trimethylammonium methyl-sulfate; ds, double-stranded; EMCV, encephalomyocarditis virus; FoxP3, forkhead box P3; Lipo, lipofectamine; MDA-5, melanoma differentiation-associated gene 5; PD, phosphodiester; poly (I:C) or pIC, polyinosine-polycytidylic acid; PTO, phosphorothioate; RIG-I, retinoic acid-inducible gene I; ss, single-stranded; Teff, T effector; Treg, regulatory T.

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synthetic long double-stranded RNA polyinosinic-polycytidylic acid [poly (I:C)] on cytosolic delivery (22).

Although the detection of viral RNA by pattern recognition receptors leads to strong immune activation, little is known about the effect of RNA ligands on Treg cell-induced immune suppression. We show in this study that RNA oligonucleotides block suppression by Treg cells in a sequence-specific and TLR7-dependent manner through inflammatory cytokines produced by APC. Infection with viruses known to stimulate the cytosolic RNA-sensing receptors RIG-I and MDA-5 blocked Treg cell function in a direct, APC-independent manner. Indeed, we show that Treg cells express RIG-I-like helicases, and activation of the MDA-5 receptor leads to an arrest of their suppressive function. Thus, suppression can be overcome both indirectly by RNA oligonucleotides activating TLR7 on APC and through direct activation of RIG-I-like helicases expressed by Treg cells.

Materials and Methods

Mice

Female BALB/c and C57BL/6 mice were purchased from Harlan-Winkelmann (Borchen, Germany). Experiments were performed using BALB/c mice unless indicated otherwise. TLR7-deficient mice were kindly provided by Prof. S. Akira (Osaka University, Osaka, Japan). MDA-5-deficient mice back-crossed to the C57BL/6 background and DERE mice expressing GFP under the control of the forkhead box P3 (FoxP3) promoter were described previously (22, 24). Mice were at least 8 wk of age at the onset of experiments. Animal studies were approved by the local regulatory agency (Regierung von Oberbayern, Munich, Germany).

Reagents

The 20-mer RNA oligonucleotides 9.2dr (5'-UGU CCU UCA AUG UCC UUC AA-3') (16), 2.2 (5'-GCA UGC GAC CUC UGU UUG A-3') (25), Poly A, and the 21-mer Poly U (26) in both the unmodified phosphodiester (PD) and fully phosphorothioated forms were obtained from CureVac (Tübingen, Germany) or Metabion (Munich, Germany). Poly (I:C) was purchased from Invivogen (Toulouse, France). The unmodified 2.2 triphosphate RNA (PPP-5'-GCA UGC GAC CUC UGU UUG A-3') (25) was produced in single- and double-strand forms by *in vitro* transcription in our laboratory (23). The PTO-modified CpG oligodeoxynucleotide 1826 (5'-TCC ATG ACG TTC CTG ACG TT-3') was obtained from the Coley Pharmaceutical Group (Langenfeld, Germany) and used at 3 µg/ml for cell stimulation. The synthetic TLR7 ligand CL097 was purchased from Invivogen and was used at 0.2 µg/ml. Anti-CD3 beads were generated by incubating monoclonal hamster anti-mouse CD3 Ab (clone 145-2C11) (BD Biosciences, Heidelberg, Germany) with tosyl-activated polystyrene beads (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions.

Flow cytometry

For flow cytometric analysis, cells were stained with anti-mouse CD4-FITC, CD25-PE, MHC-II-PE, CD11c-APC, CD69-FITC and isotype controls (BD Biosciences, Heidelberg, Germany). FoxP3 and BrdU incorporation were detected using the eBioscience Treg cell staining kit (eBioscience, San Diego, CA). After incubation with Pacific Blue-labeled FoxP3 Ab, cells were fixed with 1% paraformaldehyde in PBS, incubated with DNase I (0.05 mg/ml in PBS; Sigma-Aldrich, Steinheim, Germany) and stained with anti-BrdU-FITC Ab (Invitrogen). Analysis was performed using a FACSCanto II flow cytometer (BD Biosciences) and FlowJo software (TreeStar, Ashland, OR).

Purification of T cell subsets and APCs

CD4⁺CD25⁺ Treg cells and CD4⁺CD25^{neg} Teff cells were purified from spleen by two-step magnetic cell sorting (Miltenyi Biotec, Bergisch Gladbach, Germany). The remaining cells were used as APCs in proliferation assays (7). CD11c⁺ dendritic cells (DCs) were isolated by magnetic cell sorting and positive selection. Contamination by MHC-II⁺ APC in the purified T cell fractions was <1.5% (Supplemental Fig. 1).

Proliferation assays

T cells were cultured in RPMI 1640 medium supplemented with 10% FCS, penicillin, streptomycin, 2 mM glutamine, 2 mM sodium pyruvate, 2 mM nonessential amino acids, and 0.0001% of 2-mercaptoethanol (complete RPMI). Cultures were incubated in triplicate at 37°C and 5% CO₂ in flat-

bottom 96-well plates. CD4⁺CD25^{neg} Teff cells (7.5×10^4 cells in 200 µl) were activated with anti-CD3 beads at a bead-to-cell ratio of 1:5 in the presence of APC (2×10^4 cells), CD4⁺CD25⁺ Treg cells (5×10^4 cells, unless indicated otherwise) and the indicated ligand. T cell stimulation in the absence of APCs was performed using microbeads coated with anti-CD3 and anti-CD28 Ab (Invitrogen) (bead-to-cell ratio 1:75). Cells were then cocultured for 60 h, and BrdU (Roche Diagnostics, Mannheim, Germany) was added for the last 12 h of culture. BrdU incorporation was detected by a chemoluminescence-based assay according to the manufacturer's instructions (Roche Diagnostics) or by flow cytometry. For selected experiments, conditioned medium (CM) was produced by stimulating splenocytes with the indicated ligand for 4 h, followed by extensive washing and culture in fresh medium for an additional 20 h before removal and transfer of the supernatant into suppression assays. Where indicated, CM was preincubated with anti-IL-6 Ab (5 µg/ml; R&D Systems, Minneapolis, MN) for 4 h prior to use in suppression assays.

Cell stimulation with RNA

For endosomal targeting of TLR7, cells were transfected with RNA (final concentration of 2 µg/ml) complexed with *N*-[1-(2,3-dioleoyloxy) propyl]-*N,N,N*-trimethylammonium methyl-sulfate (DOTAP; Roche Diagnostics) according to the manufacturer's instructions. Cytosolic targeting of RIG-I and MDA-5 was achieved by adding 200 ng purified RNA oligonucleotides previously complexed in 50 µl Opti-MEM medium (Invitrogen) containing 1% Lipofectamine 2000 (Invitrogen). For stimulation of TLR3, poly (I:C) (5 µg/ml) was added directly to the culture.

Viral stimulation

EMCV was kindly provided by Dr. Anne Krug (Munich, Germany). Sendai virus (Cantell strain) was purchased from Charles River Laboratories (Wilmington, MA) (27). For stimulation with active virus, virus particles were added to the cell culture at a final concentration of 2.5 MOI (EMCV) and 0.5 MOI (Sendai Virus) for 2 h in serum-free medium and subsequently removed by repeated washing.

Quantitative PCR analysis and RNA microarray

CD4⁺CD25⁺ and CD4⁺CD25^{neg} T cells were isolated as described above. Total RNA was extracted from cells using High Pure RNA Isolation Kit (Roche Diagnostics) according to the manufacturer's instructions. One microgram of RNA was converted to cDNA using the Transcriptor High Fidelity cDNA Synthesis Kit (Roche Diagnostics). Real-time PCR amplification was performed with the LightCycler TaqMan Master (Roche Diagnostics) on a LightCycler 2.0 instrument (Roche Diagnostics) together with the Universal Probe Library System (Roche Diagnostics). Relative gene expression is shown as a ratio of the expression level of the gene of interest to that of hypoxanthine phosphoribosyltransferase RNA determined in the same sample. The primers were obtained from Metabion (Planegg, Germany). CD4⁺GFPFoxP3⁺ and CD4⁺GFPFoxP3^{neg} cells were isolated from DERE mice by negative selection of CD4⁺ T cells (Invitrogen) and subsequent FACS sorting for GFP⁺ and GFP^{neg} cells. Purity was >99%. cRNA was prepared according to the Affymetrix Labeling Protocol (Affymetrix, Santa Clara, CA), fragmented, and hybridized to Affymetrix GeneChip Mouse Genome 430 (Affymetrix). The complete microarray data set has been deposited in the GEO database at the National Center for Biotechnology Information (accession number GSE2389; www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE18387).

RNA uptake imaging by fluorescence microscopy

To detect intracellular RNA, cells were transfected with a 3'-fluorescein-labeled 9.2dr RNA (Metabion, Martinsried, Germany). After 1 h of culture, cells were fixed with 4% paraformaldehyde on poly-L-lysine coated object slides (Menzel, Braunschweig, Germany). The cell membrane and nucleus were stained at room temperature using Alexa 647-labeled cholera toxin (Invitrogen) and DAPI (Sigma-Aldrich) prior to embedding with Vectashield mounting medium (Vector Laboratories, Peterborough, U.K.). Images were obtained by fluorescence microscopy (Axiovert 2000 Carl Zeiss, Jena, Germany; 40-fold magnification) using Carl Zeiss Axiovision software and processed with Adobe Photoshop (Adobe, San Jose, CA) for adjustment of contrast and size.

Cytokine ELISA

IL-6 cytokine concentrations were determined by ELISA (BD OptEIA, BD Biosciences). Detection of IFN-α was performed by using a rat anti-mouse IFN-α monoclonal Ab (clone RMMA-1) as capture Ab followed by rabbit polyclonal

anti-mouse IFN- α as detection Ab (both from PBL Biomedical Laboratories, Piscataway, NJ). HRP-conjugated donkey anti-rabbit IgG Ab was added as secondary reagent (The Jackson Laboratory, Bar Harbor, ME) and recombinant mouse IFN- α (PBL Biomedical Laboratories) was used as standard.

Statistical analysis

All data are presented as mean. Error bars indicate SEM of $n = 3$. Analysis of variance was performed as appropriate by ANOVA or unpaired Student t test using the GraphPad Prism software (GraphPad, La Jolla, CA).

Results

Immunostimulatory RNA oligonucleotides block Treg cell-induced immune suppression in a sequence-dependent manner

To investigate whether RNA-induced immune activation influences Treg cell function, we examined a panel of RNA ligands targeting innate immune receptors for their ability to block Treg cell-induced suppression of Teff cell proliferation. We used the synthetic short RNA oligonucleotides 9.2dr, 21U, and 2.2 (16, 25, 26) to stimulate cocultures of CD4⁺CD25^{neg} Teff cells and CD4⁺CD25⁺ Treg cells

in the presence of APC and anti-CD3 Ab. Transfection of these RNAs activates innate immunity through TLR7 (16, 17). A PTO modification of the RNA backbone was used to enhance the stability and immunostimulatory capacity of these oligonucleotides (17, 28). Strikingly, transfection of the cocultured cells with the three PTO-modified sequences potently blocked Treg cell-induced suppression (Fig. 1A and Supplemental Fig. 2A). This effect was clearly sequence-specific, as an RNA of the same length containing only adenine nucleotides (Poly A) did not abolish Treg cell-induced suppression. The same RNA sequences (9.2dr and 2.2) with an unmodified PD backbone also significantly inhibited Treg cell function in a sequence-dependent manner. For all further experiments with synthetic RNA oligonucleotides, the PTO form was used. To confirm that the recovery of proliferation induced by immunostimulatory RNA in the coculture indeed resulted from sustained Teff cell proliferation, we measured BrdU incorporation in this population by flow cytometry. As expected, the Treg cell-induced decrease in the proportion of BrdU⁺ CD4⁺FoxP3^{neg} Teff cells was prevented by the addition of immunostimulatory RNA (Fig. 1B).

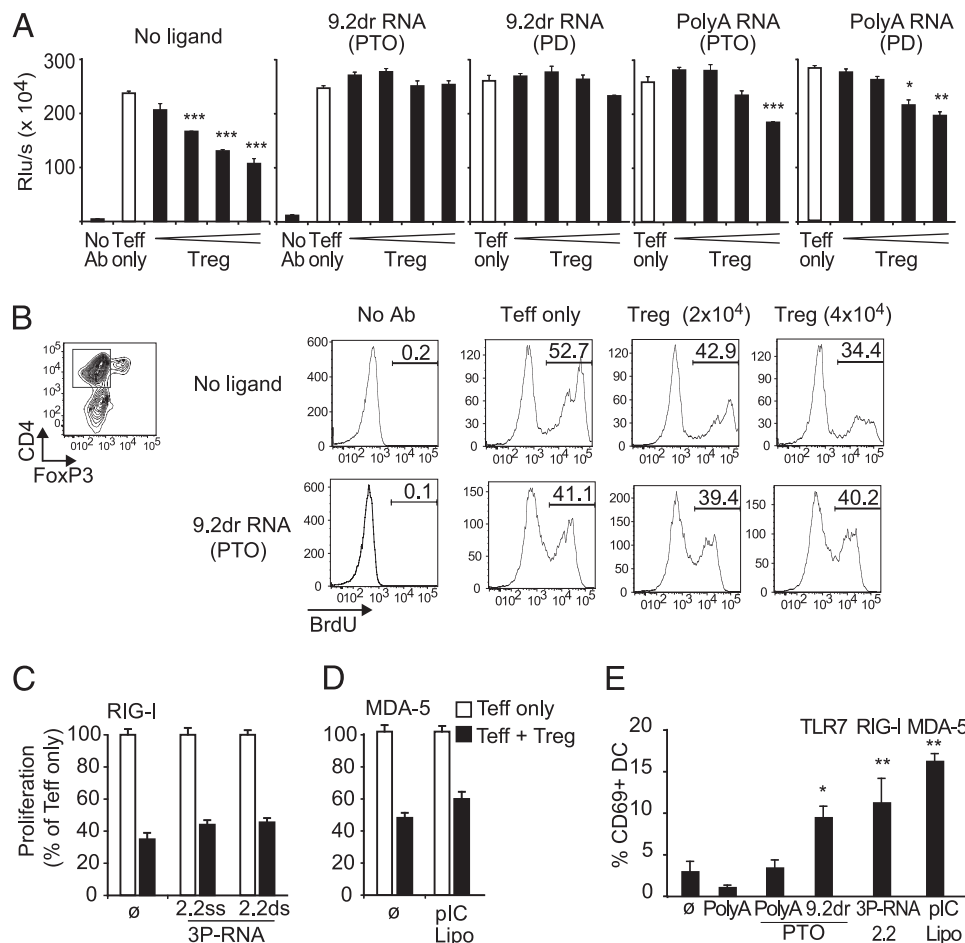


FIGURE 1. Treg cell-mediated suppression of Teff cell proliferation is blocked by synthetic RNA oligonucleotides in a sequence-dependent manner. **A** and **B**, CD4⁺CD25^{neg} murine Teff cells (7.5×10^4) were stimulated with anti-CD3 Ab (no Ab; conditions without Ab) and cultured alone (Teff only) or together with increasing numbers of CD4⁺CD25⁺ Treg cells (1, 2, 3, and 4×10^4) in the presence of APC. Cocultures were transfected with the synthetic RNA oligonucleotides 9.2dr or PolyA containing a PTO-modified (PTO) or unmodified PD backbone, using DOTAP. **A**, Proliferation of the coculture was measured on day 3 by the incorporation of BrdU using a chemoluminescence ELISA and is indicated as relative light units per second (rlu/s). Error bars indicate SEM of $n = 3$. Comparison to culture with Teff cells alone: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; no asterisk, no significant suppression. $p < 0.001$ for no ligand versus 9.2dr PTO; $p < 0.01$ for 9.2dr PTO versus Poly A PTO; $p < 0.001$ for no ligand versus 9.2dr PD; $p < 0.05$ for 9.2dr PD versus Poly A PD; no significant difference for 9.2dr PTO versus 9.2dr PD; all calculated for the highest Treg concentration. **B**, Proliferation of the coculture was measured on day 3 by flow cytometry to determine BrdU incorporation into CD4⁺FoxP3^{neg} cells. Histograms are shown for two different concentrations of Treg cells (2 and 4×10^4). **C** and **D**, Cocultures were transfected with either single-stranded (ss) or double-stranded (ds) triphosphate 2.2 RNA (3P-RNA) or poly (I:C) (pIC) using lipofectamine (Lipo) as transfection agent. **E**, 2×10^5 splenocytes were cultured with the indicated ligands for 24 h before FACS analysis of CD69 expression on CD11c⁺ DC. Error bars indicate SEM of $n = 3$. * $p < 0.05$; ** $p < 0.01$ for comparison with untreated cells. No asterisk, not significant.

Transfection of RIG-I- and MDA-5-activating RNA does not influence Treg cell-induced immune suppression

In contrast to synthetic RNA, viral RNA generally bears a triphosphate group at the 5' end and can thus activate the cytosolic RIG-I receptor, leading to activation of innate immunity (23, 29). To test the ability of triphosphate RNA to block Treg cell-induced suppression, we generated a 5'-triphosphate 2.2 RNA and targeted it to the cytosol of the cocultured cells by lipid transfection (25). Interestingly, stimulation of the cocultures by either single-stranded or double-stranded triphosphate RNA did not alter Treg cell-induced suppression (Fig. 1C). To test whether targeting RNA to the RIG-I-like helicase MDA-5 inhibits suppression of Teff proliferation by Treg cells, we performed lipotransfection of the cocultured cells with the MDA-5 ligand poly (I:C) (22). Again, we observed no changes in Teff cell suppression (Fig. 1D). Targeting TLR3 by the addition of untransfected poly (I:C) (30) also failed to inhibit Treg cell-induced suppression (Supplemental Fig. 2B). Stimulation of TLR9 with CpG DNA, however, completely abrogated Treg cell suppression as described previously (4) (Supplemental Fig. 2C). The immunostimulatory efficacy of the different RNA ligands was confirmed by analysis of the early activation marker CD69. CD69 expression on DCs was strongly upregulated by all immunostimulatory RNAs used (Fig. 1E and Supplemental Fig. 2D). In contrast, transfection of RNA without immunostimulatory sequence motifs (Poly A) did not increase CD69 levels. In summary, we observed a clear blockade of Treg cell-induced suppression by targeting TLR7 with synthetic RNAs containing immunostimulatory motifs. In contrast, no significant effects on Treg cell function were seen on liposomal transfection of ligands that stimulate RIG-I, MDA-5, or TLR3.

Synthetic RNA oligonucleotides abrogate Treg cell-induced suppression through TLR7

To test whether the block of Treg cell-induced suppression by synthetic RNA oligonucleotides indeed depended on TLR7, we stimulated cocultures of Teff cells, Treg cells, and APCs, all from TLR7-deficient mice, with the PTO-modified 9.2dr RNA. Here, in contrast to wild-type mice, no recovery of proliferation was seen on transfection with 9.2dr RNA, demonstrating that abrogation of Treg cell-induced suppression by this ligand is TLR7-dependent (Fig. 2A). The small molecule CL097, an imidazoquinoline derivative and synthetic agonist for TLR7 (31), also restored proliferation in a TLR7-dependent manner. In contrast, the TLR9 agonist CpG led to a strong recovery of proliferation in both wild-type and TLR7-deficient mice.

We next examined the importance of the transfection reagent for the immunostimulatory effect of TLR7-activating RNA. Because recognition of RNA by TLR7 takes place in the endosome (15), we selected DOTAP as the transfection reagent for its ability to deliver RNA to the endosomal compartment (32, 33). In contrast to DOTAP, lipofectamine is particularly suitable for the delivery of RNA to targets in the cytoplasm (23, 34). We show that transfection of the 9.2dr RNA with lipofectamine has no effect on Treg cell-induced suppression (Fig. 2B). This indicates a requirement for efficient endosomal delivery of RNA oligonucleotides to abrogate Treg cell-induced suppression through TLR7.

Activation of TLR7 blocks Treg cell-induced suppression in vivo

To examine whether the in vivo activation of an RNA-sensing receptor can inhibit Treg cell function, mice were injected with the TLR7 agonist CL097 or with CpG. Cells were collected from the draining lymph nodes on day 3 after initial stimulation to measure Treg cell function. Indeed, in contrast to untreated mice, the Treg cell-induced suppression was abrogated upon stimulation with CL097

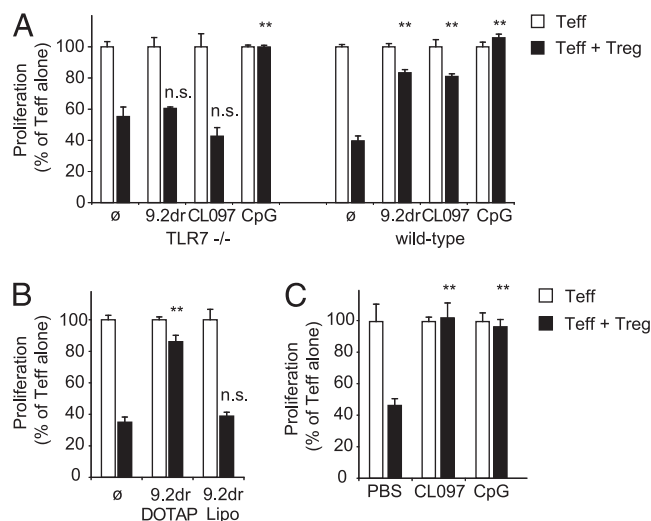


FIGURE 2. Activation of TLR7 is essential for the inhibition of Treg cell function by synthetic RNA oligonucleotides and also inhibits Treg cell-induced suppression in vivo. Teff cells were stimulated with anti-CD3 Ab and cultured alone or together with Treg cells in the presence of APC as in Fig. 1. *A*, Cells derived from either TLR7-deficient or wild-type C57BL/6 mice were stimulated with 9.2dr PTO RNA (transfected with DOTAP), the TLR7 agonist CL097, or CpG. *B*, Wild-type cocultures were transfected with 9.2dr RNA PTO using either DOTAP or lipofectamine. Error bars indicate SEM of $n = 3$. * $p < 0.05$; ** $p < 0.01$ for comparison with culture without ligands. *C*, BALB/c mice ($n = 10$) were treated with daily antebrachial injections of CL097 or CpG (both 100 μ g) for 3 d. Teff cells, Treg cells, and APCs were then isolated from the draining brachial and axillary lymph nodes of these mice and cocultured in the presence of anti-CD3 as in Fig. 1. Error bars indicate SEM of $n = 3$. ** $p < 0.01$ for comparison with culture from untreated mice.

as well as with CpG (Fig. 2C). Importantly, the fraction of FoxP3⁺ cells within the CD4⁺CD25⁺ cells remained constant after stimulation with CL097 in vivo, indicating that the loss of suppression was not due to reduced numbers of Treg cells (Supplemental Fig. 3). Thus, activation of TLR7 can overcome Treg cell-induced suppression in vivo.

Inhibition of Treg cell function through RNA oligonucleotides is not due to direct targeting of Treg cells

To examine whether synthetic RNA oligonucleotides can modulate Treg function by direct targeting of the TLR7 receptor on these cells, we cocultured wild-type Treg cells with APC and Teff cells from TLR7-deficient mice. Stimulation of these cocultured cells with 9.2dr RNA did not block the suppressive effects of the Treg cells, demonstrating the absence of a direct effect on Treg cells (Fig. 3A). This could result from poor RNA delivery to the endosomal compartment of the Treg cells. Indeed, targeting RNA to the endosome or to the cytoplasm of T cells is challenging, and lipofection-based approaches have been largely unsuccessful (35, 36). To evaluate the efficacy of RNA uptake into T cells and DCs, we transfected these cells with FITC-labeled RNA oligonucleotides followed by analysis with fluorescence microscopy. No RNA uptake was seen in T cells, whereas DC were efficiently transfected using either DOTAP or lipofectamine (Fig. 3B). Thus, lipofection efficiently delivers RNA oligonucleotides to DC but fails to target the endosome or the cytoplasm of T cells.

Abrogation of Treg cell-induced suppression by synthetic RNA oligonucleotides is mediated by APC-secreted IL-6

To determine which cell type responds to 9.2dr RNA to block Treg cell-induced suppression, we cocultured wild-type APC with

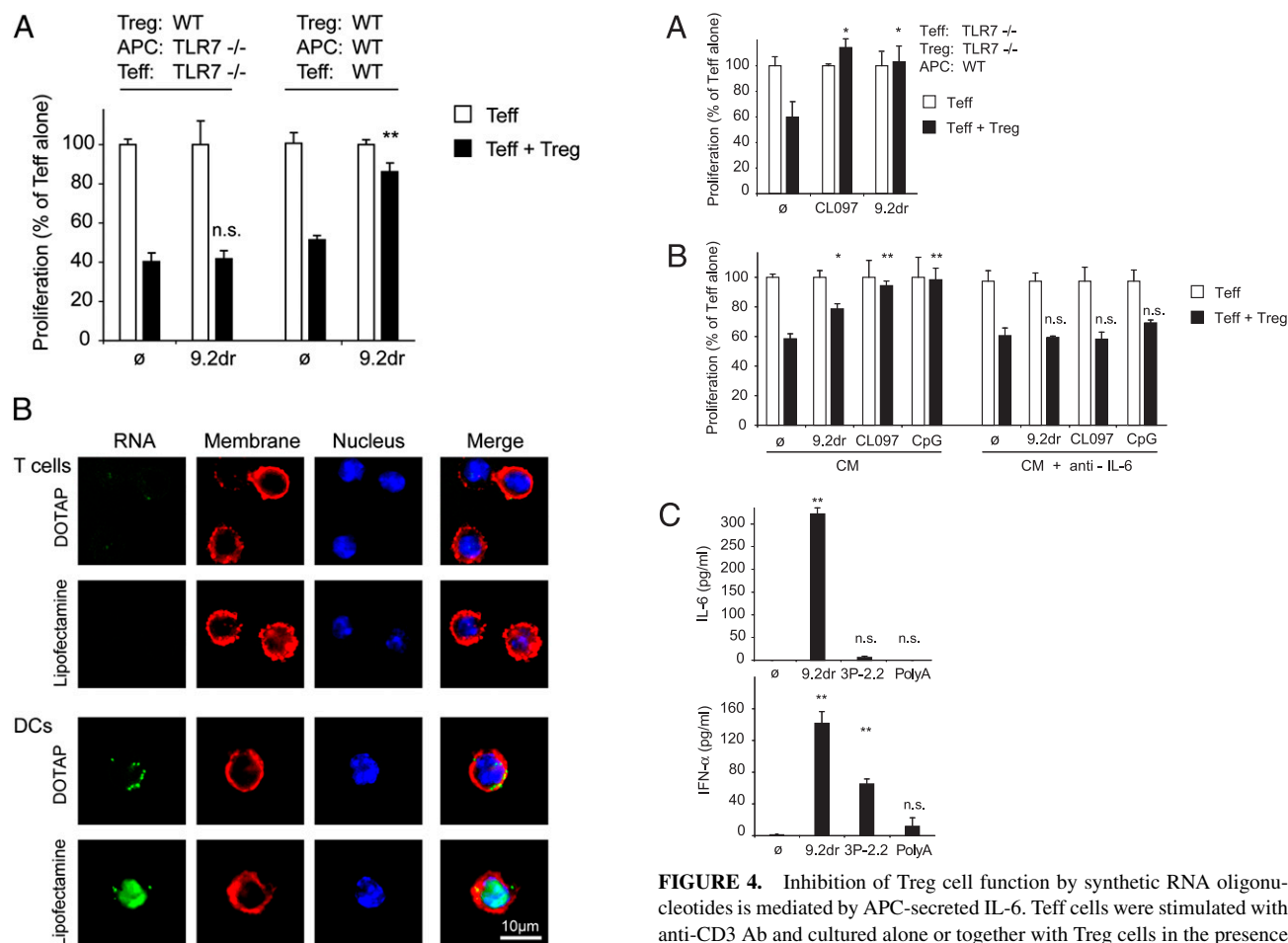


FIGURE 3. Inhibition of Treg cell function by RNA oligonucleotides is not due to direct targeting of Treg cells. *A*, Wild-type (WT) C57BL/6 Treg cells were cocultured with either TLR7-deficient or wild-type Teff cells and APC in the presence of anti-CD3 as in Fig. 1*A* and stimulated with 9.2dr PTO RNA transfected with DOTAP. Error bars indicate SEM of $n = 3$. *B*, T cells and DC were purified from the spleen by magnetic sorting prior to transfection with fluorescein-labeled (green) 9.2dr PTO RNA using either DOTAP or lipofectamine. Two hours later, cells were fixed and stained with Alexa 647-labeled cholera toxin (cell membrane dye, red) and DAPI (nucleic dye, blue). Cells were analyzed by fluorescence microscopy at a 40-fold magnification. * $p < 0.05$; ** $p < 0.01$ for comparison with culture without ligands.

TLR7-deficient T cells (both Teff and Treg cells). Even in the absence of TLR7 on T cells, both 9.2dr RNA and the synthetic ligand CL097 completely blocked Treg cell-induced suppression (Fig. 4*A*), indicating that the inhibition of Treg cell suppression by synthetic RNA oligonucleotides is mediated by APC. To determine whether the inhibition of Treg cell function is mediated by a soluble factor produced by APC, we stimulated cocultures of Teff and Treg cells with supernatant from 9.2dr RNA-activated APC. To ensure the absence of the ligand from the supernatant, supernatants were generated by stimulation of APC with RNA for 2 h, thorough washing of the cells, and culture for another 12 h in fresh medium. The resulting CM was sufficient to reverse Treg cell-induced suppression (Fig. 4*B*). In contrast, addition of CM generated by APC from TLR7-deficient mice did not lead to recovery of proliferation (data not shown). As the proinflammatory cytokine IL-6 is essential for protection from Treg cell-induced suppression by CpG and LPS (4), we tested whether blocking IL-6 in the 9.2dr RNA CM would impact the reversal of Treg cell-induced suppression. Indeed, neutralization of IL-6 completely prevented the

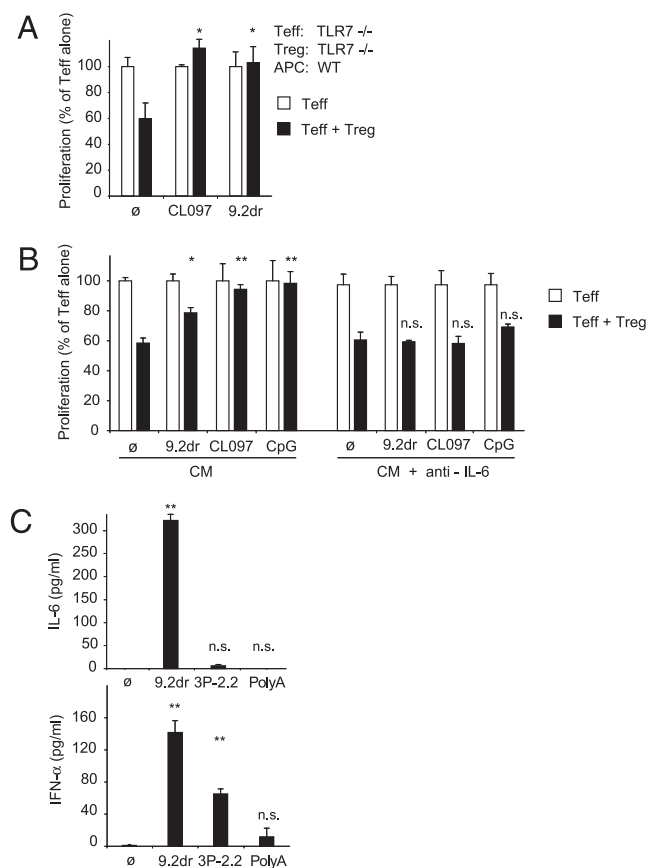


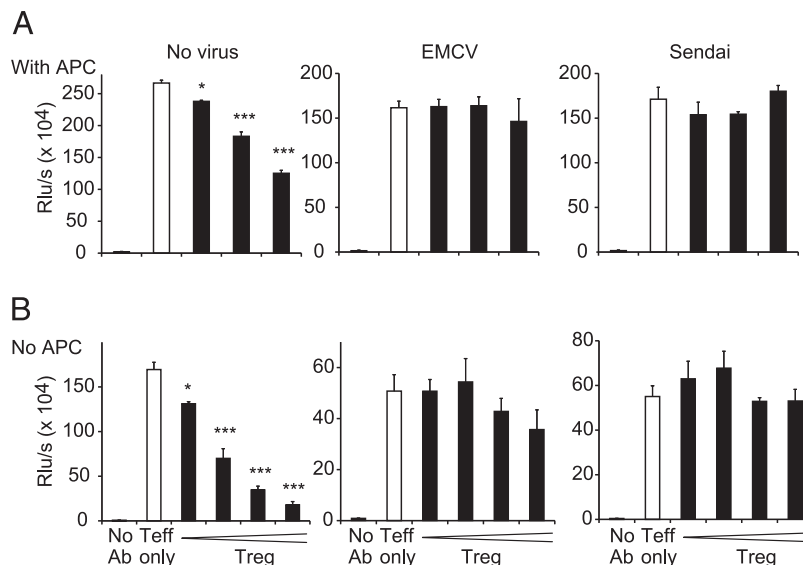
FIGURE 4. Inhibition of Treg cell function by synthetic RNA oligonucleotides is mediated by APC-secreted IL-6. Teff cells were stimulated with anti-CD3 Ab and cultured alone or together with Treg cells in the presence of APC as in Fig. 1*A*. *A*, TLR7-deficient Teff and Treg cells were cocultured with wild-type (WT) C57BL/6 APC and stimulated with 9.2dr PTO RNA (transfected with DOTAP) or the synthetic TLR7 agonist CL097. *B*, Wild-type Teff cells, Treg cells, and APC were cultured in CM either with or without anti-IL-6 Ab. CM was generated by incubating wild-type APC with RNA 9.2dr, CL097, or CpG for 2 h followed by extensive washing and culture in fresh medium without additional stimulation for 24 h. *C*, 2×10^5 splenic APC were stimulated for 24 h with either 9.2dr PTO RNA (transfected with DOTAP), 5'-triphosphate RNA (3P-2.2, transfected with lipofectamine), or Poly A PTO (transfected with DOTAP) and the levels of IL-6 and IFN-α were determined by ELISA. Error bars indicate SEM of $n = 3$. * $p < 0.05$; ** $p < 0.01$ for comparison with culture without ligands.

block of Treg suppression by RNA oligonucleotides (Fig. 4*B*). We next assessed the efficacy of the RNA ligands to induce IL-6 production in splenic APC. Consistent with its capacity to block Treg cell suppression, 9.2dr RNA induced the highest levels of IL-6 (Fig. 4*C*). Interestingly, targeting RIG-I with 5'-triphosphate RNA did not lead to secretion of IL-6, although the proinflammatory cytokine IFN-α was clearly induced (Fig. 4*C*). The absence of IL-6 production on stimulation with 5'-triphosphate RNA may thus explain its inability to block Treg cell function.

RNA viruses inhibit Treg cell function independently from APC

The RNA-sensing receptors MDA-5 and RIG-I can be specifically activated by distinct viruses: EMCV exclusively activates MDA-5, whereas the Sendai virus specifically stimulates RIG-I (22, 37). To examine the effect of viral infection on Treg cell-mediated suppression of Teff cell proliferation, we infected cocultures of Teff and Treg cells with these viruses. Strikingly, both in the presence and in the absence of APC, the suppression of proliferation by Treg cells was blocked on viral infection (Fig. 5*A*, 5*B*). Inhibition

FIGURE 5. RNA viruses inhibit Treg cell function independently from APC. Teff cells (7.5×10^4) were cocultured in the presence (A) or in the absence (B) of APC with increasing concentrations of Treg cells (1.25 , 2.5 , 3.75 , and 5×10^4 ; no 5×10^4 condition for A) for 3 d. Cocultures were stimulated with microbeads coated with anti-CD3 and anti-CD28 Ab and infected with EMCV and Sendai virus. No Ab, conditions without Ab-coated beads. Error bars indicate SEM of $n = 3$. For comparison with culture with Teff cells alone: $*p < 0.05$; $***p < 0.001$; no asterisk, no significant suppression.



of Treg cell function seemed to be most effective upon infection with Sendai virus. Generally, viral infection led to a moderate reduction in the overall proliferation levels that may result from a reduction of cellularity. Flow cytometry analysis confirmed that $CD4^+FoxP3^{neg}$ Teff cells show sustained proliferation on viral infection (Supplemental Fig. 4); the proliferation levels of Treg cells remained unaffected (data not shown). Viral infection did not lead to IL-6 production in the T cell coculture (IL-6 was not detectable for both EMCV and Sendai virus), confirming T cell purity and thus an APC-independent mechanism for the inhibition of Treg cell function. Thus, RNA viruses can inhibit the suppressive function of Treg cells even in the absence of APC.

Treg cells express the RNA-sensing receptors RIG-I and MDA-5

The direct effect of RNA viruses on Treg cell function suggested that T cells may express RNA-sensing receptors. To analyze Treg and Teff cells for their expression of RIG-I-like helicases $CD4^+CD25^+$ Treg cells and $CD4^+CD25^{neg}$ Teff cells were isolated by magnetic cell separation from the spleen of wild-type BALB/c mice and analyzed by quantitative PCR. Interestingly, Treg and Teff cells expressed both RIG-I and MDA-5 at levels similar to DC (Fig. 6A). Relative to expression levels on DC, RIG-I was expressed on Treg cells at higher levels than MDA-5. Analysis of TLRs revealed low expression of TLR9 on both Treg and Teff cells compared with DC, whereas TLR7 expression was low only on Teff cells. Analysis by mRNA microarray from highly pure $CD4^{GFP}FoxP3^+$ and $CD4^{GFP}FoxP3^{neg}$ cells sorted from mice expressing GFP under the control of the FoxP3 promoter (24) confirmed the expression of RIG-I and MDA-5 on Treg cells (Fig. 6B).

Loss of Treg cell function upon EMCV infection is mediated through MDA-5

The virus-induced APC-independent loss of Treg cell-mediated suppression and the presence of RNA-sensing receptors on these cells suggests that activation of RIG-I-like helicases in Treg cells may directly block their function. To test this hypothesis, we purified Treg and Teff cells from wild-type and MDA-5-deficient mice and infected the cocultures with EMCV. EMCV infection did not alter Treg cell function in the cocultures derived from MDA-5-deficient mice, in contrast to inhibition of Treg cell function seen in wild-type mice (Fig. 7, top and middle panels). To investigate whether MDA-5 expression on Treg cells is required to

inhibit their suppressive function, we cocultured MDA-5-deficient Treg cells together with wild-type Teff cells and infected the cells with EMCV. Strikingly, the selective deficiency of MDA-5 on Treg cells was sufficient to completely prevent the virus-induced loss of Treg cell function (Fig. 7, lower panel). Thus, activation of MDA-5 on Treg cells is necessary to block their suppressive function on viral infection. Interestingly, by coculturing wild-type Treg cells together with MDA-5-deficient Teff cells, we found that MDA-5 expression by Teff cells is a further requirement for the loss of Treg cell function (Supplemental Fig. 5). Thus, inhibition of Treg cell function by EMCV requires that both Treg and Teff cells are simultaneously stimulated through MDA-5.

Discussion

Viral infection of the host is generally followed by the induction of antiviral immunity. The development of an immune response is, however, tightly controlled by Treg cells, and the mechanisms by which Treg cell-induced suppression is overcome on viral infection remain incompletely understood. As viral nucleic acids are potent activators of innate immunity, we investigated the potential of RNA ligands to inhibit Treg cell function. Interestingly, only the endosomal delivery of PTO-modified RNA oligonucleotides with specific immunostimulatory sequence motifs (9.2dr, 21U or 2.2) blocked Treg cell-induced suppression. This effect was mediated through stimulation of TLR7, as suppression was not inhibited in TLR7-deficient mice. Cytosolic targeting of RIG-I-like helicases with 5'-triphosphate RNA or poly (I:C) elicited strong activation in terms of cytokine secretion or upregulation of the early activation marker CD69 on DCs, but failed to block Treg cell-induced suppression. Inhibition of Treg cell function through TLR7 was mediated indirectly via soluble factors produced by APC. Neutralization of IL-6 completely prevented this effect, as shown previously for the bacterial ligands LPS and CpG (4). Among the different immunostimulatory RNAs tested in our study, the TLR7-activating RNA oligonucleotides induced strong IL-6 production, which has been shown to require MyD88-dependent activation of NF- κ B (38, 39). Although activation of RIG-I can also signal via NF- κ B through the adaptors IFN- β promoter stimulator protein 1 and TNFR-associated factor-6 (40, 41), we show that, on stimulation of primary murine splenocytes with triphosphate RNA, only low levels of IL-6 are produced, in contrast to high levels of type I IFN. This may thus explain the inability of triphosphate RNA to overcome Treg cell-induced suppression.

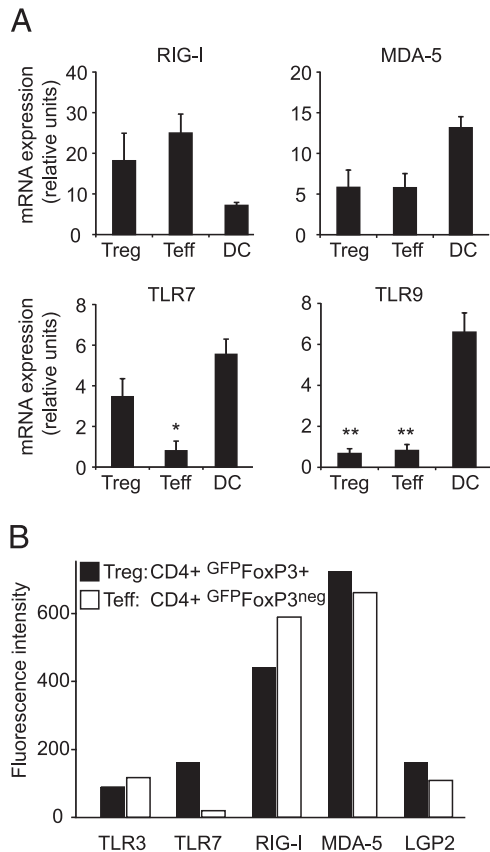


FIGURE 6. Treg cells express RIG-I-like helicases. *A*, Real-time PCR analysis of RIG-I, MDA-5, TLR7, and TLR9 transcript patterns in CD4⁺ CD25⁺ Treg cells, CD4⁺CD25^{neg} Teff cells, and CD11c⁺ DCs. Levels of mRNA are represented as relative units reflecting the copies per hypoxanthine phosphoribosyltransferase transcript. Error bars indicate SEM of $n = 3$. Comparison to DC: * $p < 0.05$; ** $p < 0.01$; no asterisk, no significant difference to DC. *B*, CD4⁺GFP⁺FoxP3⁺ Treg cells and CD4⁺GFP⁺FoxP3^{neg} Teff cells were purified from mice expressing GFP under the control of the FoxP3 promoter. The mRNA levels of both cell types were analyzed by microarray. Bars indicate the fluorescence intensity of the respective gene transcripts.

Inhibition of Treg cell function is an important issue for many clinical applications. During vaccination, any type of adjuvant needs to bear the potential to overcome Treg cell suppression (42). We show in this study that the immunostimulatory capacity of the different RNAs in terms of induction of CD69 or IFN- α secretion does not necessarily correlate with their potential to suppress Treg cell function. In particular, transfection of DCs with triphosphate RNA led to a potent activation of the cells, but did not alter suppression by Treg cells. In vivo, this type of RNA has also been proven to potentially activate innate immunity (25), but its potential as a vaccine adjuvant remains to be shown. We further demonstrate in this study a high efficacy of synthetic PTO-modified short immunostimulatory RNA for the reversal of Treg cell suppression. Indeed, these TLR7-activating RNAs have been proven to be effective adjuvants for the induction of Ag-specific T and B cells (17, 43, 44). Immunosuppression by Treg cells plays a crucial role in the process of malignant disease, and strategies aiming to inhibit these cells in cancer-bearing organisms improve the efficiency of immunotherapy (45, 46). Suppression of Treg cell function increases host immunity against cancer and efficient delivery of ligands for TLR7 or RIG-I-like helicases may thus represent a promising therapeutic approach.

The suppressive function of Treg cells can be regulated not only indirectly by inflammatory factors derived from APC, but also

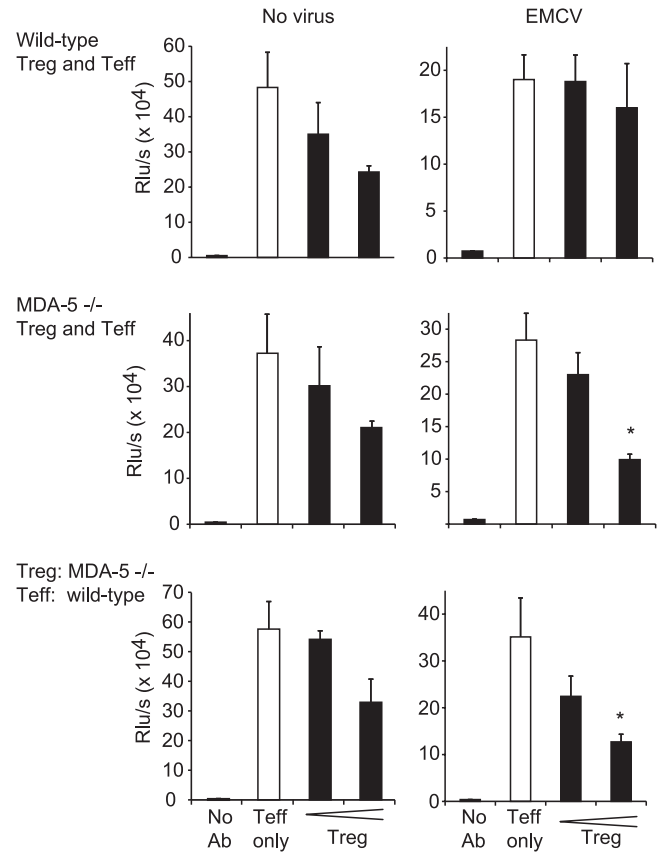


FIGURE 7. Inhibition of Treg function on EMCV infection depends on MDA-5 expression in Treg cells. Teff cells (7.5×10^4) from wild-type C57BL/6 or MDA-5-deficient mice were stimulated using microbeads coated with anti-CD3 and anti-CD28 Ab and cultured alone or together with increasing concentrations of Treg cells (1.25 and 2.5×10^4) in the absence of APC. Cocultures were stimulated with EMCV. No Ab, conditions without Ab-coated beads. Error bars indicate SEM of $n = 3$. * $p < 0.05$ for comparison with culture with Teff cells alone.

through the direct activation of TLRs expressed on these cells themselves (47). A semiquantitative RNA analysis of murine Treg cells previously showed a strong expression of the TLRs 1, 2, 4, and 7 and lower levels of TLR5 and 8 (48); expression of TLR2 was further confirmed by quantitative PCR (9). We show in this study for the first time that Treg cells express not only TLRs but also the cytoplasmic helicases RIG-I and MDA-5, and we confirm these novel findings on highly purified GFP⁺FoxP3⁺ cells. Liposomal transfection of the cells with RNA ligands for RIG-I and MDA-5, however, showed no significant effects on Treg cell suppression. This may be due to a limited transfection efficacy of primary T cells (35, 36). Indeed, we could show that liposomal reagents failed to deliver RNA ligands to the cytosol or to the endosome of T cells, but efficiently targeted both compartments in DCs. An alternative method of nucleic acid delivery is the transduction of cells by viruses. In this study, we used viruses known for their specific ability to stimulate either RIG-I (Sendai virus) or MDA-5 (EMCV). Interestingly, both viruses completely reversed Treg cell-induced suppression even in the absence of APC and in an IL-6-independent manner. In a previous study, transfer of supernatant from DCs stimulated with inactivated Sendai virus particles could reduce Treg cell suppression (49), indicating that viruses could also indirectly inhibit the function of Treg cells. We show in an APC-independent system that direct stimulation of the RIG-I-like helicase MDA-5 on Treg cells is necessary to inhibit their suppressive function upon viral infection. In addition, the

simultaneous activation of MDA-5 on Teff cells is required for this inhibition. This suggests that activation by EMCV initiates interactions between Treg and Teff cells that block Treg cell-induced suppression. In summary, RIG-I-like helicases expressed by Treg and Teff cells play a previously unidentified role in modulating T cell activation and may represent an important component in the regulation of the host immune response.

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Disclosures

The authors have no financial conflicts of interest.

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